Crop Breeding and Applied Biotechnology 9: 239-245, 2009
Brazilian Society of Plant Breeding. Printed in Brazil

DNA marker-assisted evaluation of cultivated and local mulberry genotypes of southern India

Keshava Murthy Bengaluru Channappa[1,2*], Bandekodigenahalli Marappa Prakash[2,3], Shailaja Hittalmani[1], and Hosagavi Puttegowda Puttaraju[2]

Received 25 March 2008

Accepted 09 April 2009

ABSTRACT - *Germplasm evaluation is essential in any crop improvement program and genetic characterization at morphological and molecular level is very vital for breeding programs to be successful. Twenty six cultivated and local genotypes of mulberry were subjected to diversity analysis with RAPD markers. Among the total 31 RAPD primers studied, 24 were polymorphic and 7 were monomorphic. Of the total 197 loci obtained from 24 polymorphic primers, 110 loci (55.83%) were polymorphic and 87 (44.16%) were monomorphic. A clear grouping was seen among the cultivated genotypes based on yield with varieties like S36, V1, S54 and M5 showing proximity to each other and sharing a close similarity. Local genotypes are rich reservoirs of resistant gene sources and are well acclimatized to the prevailing environmental conditions. Utilization of these along with other high yielding varieties will produce various combinations of resistance sources which can be incorporated into high yielding varieties.*

Key words: Local genotypes, diversity, polymorphism, dendrogram, DNA marker.

INTRODUCTION

Among different tree species cultivated in India, mulberry is one of the important economic plants cultivated for the production of leaves to rear silkworm. The quality of mulberry leaves becomes very vital for the growth of silkworm, as the essential major and minor nutrients are derived from the leaves. Production of quality leaves directly depends on cultivated varieties, which are able to sustain the adverse conditions of the environment and provide maximum leaf yield with good water retention capacity. Although many varieties have been developed for tropical and temperate climatic conditions, a sustainable variety for both favorable and adverse situations is in need of the hour. Trees usually have high levels of isozyme variation, which makes them quite adequate for germplasm identification problems. One limitation of isozymes is that a large number of loci cannot be resolved from some tissues. This limitation could be overcome with DNA markers and the first, and probably most important, advantage is that potentially an unlimited number of DNA markers can be detected and DNA markers do not vary among tissue types or developmental stages of the plant because the assays are based on the DNA itself and not the products of genes. A third advantage of DNA markers is that they are not affected by environmental variation (Neale et al. 1992).

Germplasm resources are very much required for the continuous improvement of crop plants, where genetic characterization is essential for scientific germplasm conservation and characterization permits the estimation of genetic relatedness and diversity (Bhat and Jarret 1995). Although vast genetic resources are

[1] Marker Assisted Selection Laboratory, Department of Genetics and Plant Breeding, University of Agricultural Sciences, G.K.V.K. Bangalore-560 065, India. *E-mail: drmurthy_mb@rediffmail.com
[2] Laboratory of Seri-Biotechnology, Department of Biological Sciences (Integrated), Bangalore University, Bangalore-560 056, India
[3] Evolutionary Biology Laboratory, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore-560 064, India

available for mulberry, until now very few of them have been used in breeding programs. Molecular markers are very effective in germplasm evaluations for differentiating cultivars and for screening for novel/superior genes (alleles) and constructing a representative subset or core collection (Xu et al. 2003). Genetic diversity provides the raw material for breeding and plant improvement. This is often the prime importance and the occurrence of particularly desirable genes that are of great interest to plant breeders (Treuren and Hintum 2003).

The polymerase chain reaction based genotyping methods have been extensively used for diversity analysis in many crop species. The random amplified polymorphic DNA (RAPD) method is very economical and allows the detection of polymorphism without prior knowledge of nucleotide sequences. This marker has been widely used for diversity analysis and was developed by Williams et al. (1990). RAPD markers have been used to study the genetic relationship in a number of tree species (Krabel et al. 1998, Casas et al. 1999, Ravishankar et al. 2000, Venkateswarulu et al. 2006, Rao et al. 2008) and even mulberry plants have been subjected to analysis to study the genetic relationship of cultivars with AFLP, RAPD, ISSR and SSR markers (Sharma et al. 2000, Aggarwal et al. 2004, Vijayan et al. 2004, 2006).

Mulberry silk is mostly produced in the southern regions of India and genotypes have been cultivated as irrigated and rainfed cultivars. These cultivars cover a maximum area of commercial cultivation and are acclimatized to the local prevailing environmental conditions. The objective of this study was to evaluate a group of cultivated varieties along with some local landraces for genetic diversity and identification of suitable genotypes for the development of segregating populations.

MATERIAL AND METHODS

Plant materials

Twenty six mulberry genotypes including mostly cultivated varieties and local accessions were selected for this study. The genotypes were collected from the germplasm bank at the Department of Sericulture, Bangalore University, Bangalore, India.

DNA extraction

Fresh mulberry leaves were collected in the morning hours and DNA was isolated by the modified CTAB (Cetyl-trimethyl ammonium bromide) protocol method of Murray and Thompson (1980). About 50 grams of leaves were ground in liquid nitrogen using mortar and pestle. The leaf powder was transferred into a 15mL falcon tube containing 6mL of extraction buffer (1% CTAB, 1.5% PVP, 1.4M Nacl, 50mM EDTA, 50mM Tris-HCl, 0.1% β-mercaptoethanol) and was incubated in a water bath at 65 °C. After incubation, equal volumes of phenol: chloroform (1:1) was added to the incubation mixture and thoroughly mixed and centrifuged at 10,000 rpm in a centrifuge at 25 °C for 15 min. To the supernatant, an equal volume of phenol: chloroform: Iso-amylo alcohol (24:24:1) was added and centrifuged again at 10,000 rpm for 15 minutes. The supernatant was then transferred to a fresh tube and an equal volume of chloroform was added and mixed thoroughly by inverting the tubes. The mixture was further centrifuged at 10,000 rpm for 15 minutes. Adding one-tenth the volume of 3M-sodium acetate and two volumes of ethyl alcohol precipitated the DNA from the supernatant. The precipitated DNA was washed with 70% ethanol and air dried at 37 °C. The DNA was later dissolved in a TE buffer (10mM Tris-HCl-pH 8.0 and 1M EDTA). Contamination of RNA was removed by incubating the dissolved DNA with DNAase free bovine pancreatic RNAase (10 mg mL^{-1}) at 37 °C for 1 h. The DNA was then re-extracted following the steps stated above. Quantification of the DNA was performed by electrophoresis in 0.8% agarose gel (1X TAE) stained with Ethidium bromide (0.5 μg mL^{-1}) and using uncut λ DNA (10 ng μL^{-1}) as a standard.

PCR analysis with RAPD primers

A total of 31 selected decamer primers designed by the Operon Technologies Inc USA, were used in this study. The PCR amplification was carried out in an Eppendorf thermal Cycler (96 welled) according to Williams et al. (1990). The PCR reaction mixture consisted of a 50ng template DNA, 20 pmol of each of the primers, 0.1 mM dNTP's, 1X PCR buffer (10mM Tris, pH 8.0, 50mM KCl, 18 mM $MgCl_2$ and 0.01mg mL^{-1} gelatin) and 1 unit of *Taq* polymerase in a 20μL volume. Template DNA was initially denatured at 94 °C for 5 min, followed by 35 cycles of 94 °C for a 1min denaturation, primer annealing at 36°C for a 1 min and 2 min primer extension at 72 °C. The final 5 min incubation at 72 °C was allowed for completion of the primer extension. The amplified products were electrophoretically resolved in 1.4% agarose in a 1X TAE buffer.

Statistical analysis

Each amplified loci were considered as a unit character and were scored as 1 for presence and 0 for absence. A binary matrix of 0 and 1 was constructed on these underlying data. The presence of one common band between the two compared genotypes was considered as similarity and the presence in one genotype and absence in the other as difference. Genetic distance (dissimilarity) was calculated pair wise, considering only the polymorphic bands, using the arithmetic complement of the indices of similarity of Jaccard (JAC) and Sorenso-Nei and Li (SNL) and cluster analysis was carried out using UPGMA-Unweighted pair-group method arithmetic average (Sneath and Sokal 1973) using STATISTICA 4.5.

RESULTS AND DISCUSSION

It is evident from the phenotypic data that a larger genetic variation exists in mulberry with variations in leaf length in a range of 10.76-18.06 cm and leaf width from 7.88-16.20 cm. Variations for yield among the different genotypes of the present study were ranging from 8,000-60,000 kg ha year^{-1}. Molecular analysis with RAPD markers further confirmed polymorphism among the studied varieties (Figure 1). Among the total 31 RAPD primers analyzed, 24 were polymorphic and seven were monomorphic. A total of about 197 loci were obtained and among which 110 loci (55.83%) were polymorphic and 87 (44.16%) were monomorphic. The number of polymorphic loci obtained in the present study was smaller when compared to earlier studies of Bhattacharya et al. (2001) and Awasthi et al. (2004) and this may be due to the lack of suitable binding sites of the primers used. An average of about 6.3 bands was generated per primer, which is higher than the number of bands obtained in earlier studies. The fragment size varied from 0.2-5kb for the amplified loci obtained and primers OPA-10, OPB-17, OPC-03 and OPC-04 produced a maximum of 11 loci per each primer studied. A maximum of 14 loci per RAPD primer was generated with primers OPB-08 and OPO-19 (Table 1, Figure 1-a). Since RAPD markers randomly scatter throughout the genome, the number of markers matters greatly in analyzing the accessions that would cover the entire genome to a greater extent

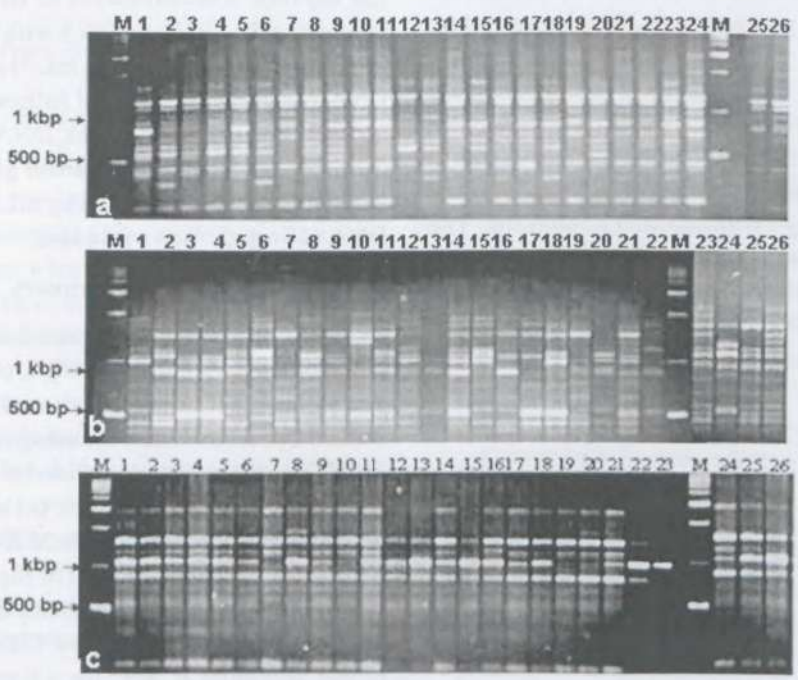

Figure 1. Electrophoretic analysis in agarose gels of selected polymorphic RAPD primers. a- Profiles obtained for primer OPO-19, Lanes: M: Molecular weight marker; lane 1 to 24 mulberry varieties; M: Molecular weight marker; Varieties 25 and 26. b- Maximum number of bands with primer OPB-08, Lanes: M: Molecular weight marker; lane 1 to 22 mulberry varieties; M: Molecular weight marker; Varieties 23, 24, 25 and 26. c- Polymorphic loci obtained with primer OPB-13, Lanes: M: Molecular weight marker; lane 1 to 23 mulberry varieties; M: Molecular weight marker; Varieties 24, 25 and 26

Table 1. List of RAPD primers and number of PCR amplified bands generated across 26 mulberry genotypes

Sl. No.	Primer	Number of bands		
		Total	Monomorphic	polymorphic
1	OPB-17	11	0	11
2	OPB-18	10	6	4
3	OPA-14	6	3	3
4	OPA-10	11	8	3
5	OPA-15	9	3	6
6	OPB-13	9	3	5
7	OPB-14	6	2	4
8	OPC-01	6	2	4
9	OPC-02	6	3	3
10	OPC-03	11	8	3
11	OPC-04	11	6	5
12	OPC-07	7	3	4
13	OPC-08	9	7	2
14	OPO-19	13	4	9
15	OPB-08	14	3	11
16	OPB-05	6	2	4
17	OPB-06	9	2	7
18	OPB-11	9	4	5
19	OPB-12	4	2	2
20	OPB-13	4	2	2
21	OPB-14	6	4	2
22	OPO-08	9	2	7
23	OPO-09	6	3	3
24	OPA-11	7	5	1
Total		197	87	110

(Williams et al. 1990).

The dendrogram generated based on the UPGMA analysis from the genotypic data clearly grouped mulberry genotypes into two broad clusters at 44% dissimilarity. The S30, RFS175, S13, C11 and Mysore local varieties formed one main cluster and the remaining 21 genotypes grouped into another main cluster. This cluster was again divided into 2 sub clusters at 33% dissimilarity and joined to another cluster with 3 varieties at 40% dissimilarity. The S54 and S36 varieties shared very low variability and formed a cluster at 11% dissimilarity (Figure 2). The similarity among 26 mulberry genotypes showed a minimum range of 0.11 between S36 and S54 and a maximum range of 0.70 between C11 and DD varieties (Table 2). There was a clear grouping between the cultivated genotypes and local genotypes. Mostly cultivated varieties like S36, V1, S54 and M5 were very close to each other and shared similarity backgrounds. These four genotypes occupy a maximum acreage of mulberry cultivation in southern India and have high yields, demonstrating to be highly responsive to agronomic inputs and have been recommended for irrigated regions. The high yielding varieties, S36 and S54, were developed by chemical mutagenesis from a local variety from west Bengal and are similar with respect to most of the phenotypic traits and the dendrogram also depicted the same close

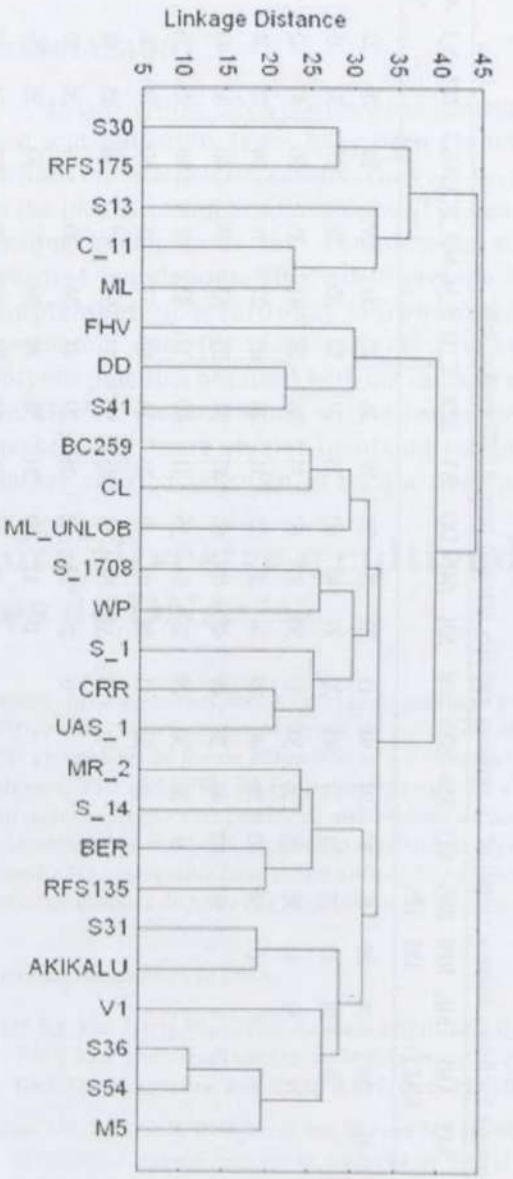

Figure 2. Dendrogram constructed using the unweighted pair-group method arithmetic average with 24 polymorphic RAPD primers for 26 varieties

Table 2. Squared Euclidean distance (x100) of 26 mulberry varieties obtained from RAPD marker data

	M5	AKI KALU	ML	RFS 135	RFS 175	S13	S54	S30	S36	S31	S41	DD	V1	BER	C_11	S_14	MR_2	UAS_1	CRR	S_1	ML_U NLOB	FHV	CL	BC 259	WP	S_ 1708
M5	0	29	36	20	46	30	24	44	15	22	27	33	29	26	47	31	29	24	29	22	29	28	33	32	34	34
AKIKALU		0	21	37	37	31	31	41	32	19	48	60	28	27	32	30	28	27	32	35	30	41	36	31	47	31
ML			0	44	36	28	36	38	35	30	57	67	35	36	23	41	35	36	47	42	43	54	47	42	52	40
RFS135				0	54	40	36	48	29	34	23	35	31	20	45	27	31	24	25	28	27	26	27	34	30	26
RFS175					0	40	46	28	49	42	65	63	47	52	35	53	45	46	53	40	49	60	53	52	52	38
S13						0	32	36	29	28	51	55	41	40	37	43	41	36	33	36	41	50	45	42	40	36
S54							0	38	11	28	43	49	25	34	41	33	25	30	35	34	39	40	33	34	36	36
S30								0	43	38	61	63	45	50	33	51	47	46	45	44	45	56	51	46	50	36
S36									0	27	34	42	26	29	44	36	30	33	36	33	36	37	38	35	33	39
S31										0	41	49	31	36	39	39	37	30	35	36	37	36	39	36	44	40
S41											0	22	42	37	62	44	42	37	34	37	38	27	38	37	37	45
DD												0	56	47	70	48	46	45	46	41	52	33	48	47	33	49
V1													0	27	36	34	32	31	28	35	34	33	34	29	41	33
BER														0	31	23	21	30	29	28	27	34	29	26	38	30
C_11															0	42	36	47	48	45	36	57	44	43	57	33
S_14																0	24	33	36	35	36	33	34	39	41	33
MR_2																	0	33	36	27	34	33	34	35	35	31
UAS_1																		0	21	26	31	34	33	28	32	28
CRR																			0	25	30	33	32	27	33	31
S_1																				0	33	36	27	32	28	28
ML_UNLOB																					0	35	28	31	41	27
FHV																						0	33	38	36	38
CL																							0	25	37	31
BC259																								0	36	32
WP																									0	26
S_1708																										0

relatedness between these two varieties. In earlier studies V1 has been grouped closely with the rainfed variety RFS175 (Vijayan et al. 2004) but in the present study V1 was grouped with the high yielding varieties S54, S36 and M5. In another group local genotypes like Channapatna local, Mysore local unlobed and Berhampore, which are known for marginal resistance to drought conditions were very close to each other and shared a genetic distance from 0.28 to 0.30. Such results of relatedness for both genotype and phenotype proved the accuracy of the DNA marker utilization in estimating genetic relationships, which is devoid of environmental influence. Genotypic data obtained in the present study also grouped these varieties very closely and this shows the efficiency of the RAPD marker in evaluating the plant accessions.

The mulberry genome is coupled with a long generation time with difficulties in generating large segregating progeny populations and a small mistake in selection will lead to a huge loss of time and resources. From earlier morphological studies (Fotadar and Dandin 1998) it has been proved that the mulberry genome exhibits a rich diversity and by utilizing DNA markers this data will be further validated. The present study suggests that cultivated varieties like S36, V1, S54 and rainfed varieties like Mysore local, Channapatna local or Berhampore local should be extensively used in the breeding programs. Among all the markers used for genetic diversity studies, the RAPD marker appears to be a very easy and highly economical method for diversity studies in various animal and plant genomes. Although repeatability of the amplified loci is very poor, generation of genetic markers at low cost in much less time makes them very suitable for germplasm evaluation and varietal identification.

CONCLUSION

In conclusion, DNA marker advancement is very vast and numerous types have been invented and utilized on different organisms. They are very useful in the identification and development of genetically unique germplasms that complement existing breeding populations. This would provide a useful supplement to traditional morphological and agronomic data for plant varietal development. Polymorphic loci obtained with the random markers are also very useful along with other co-dominant markers for future studies involving mapping and marker assisted selection in such a tree species.

Avaliação de genótipos de amora silvestre e cultivada no Sul da Índia por marcadores de DNA

RESUMO - *A avaliação do germoplasma ao nível morfológico e molecular é essencial para o sucesso de qualquer programa de melhoramento genético de plantas. Vinte e seis genótipos silvestres e cultivares de amora foram submetidos à análise de diversidade com marcadores RAPD. Do total de 31 primers RAPD estudados, 24 foram polimórficos e 7 monomórficos. A partir dos 24 primers polimórficos foram obtidos 197 locos; desses, 110 (55,83%) foram polimórficos e 87 (44,16%) monomórficos. Um agrupamento claro com base no rendimento foi observado entre os genótipos cultivados e as variedades S36, V1, S54 e M5, mostrando proximidade entre eles e uma estreita semelhança genotípica. Os genótipos silvestres representam uma rica fonte de genes de resistência e são bem aclimatados nas condições ambientais locais. Sua utilização em combinação com outras variedades de alto rendimento pode produzir diferentes combinações de fontes de resistência, as quais podem ser incorporadas em variedades de alta produtividade.*

Palavras-chave: Genótipos locais, diversidade, polimorfismo, dendrograma, marcadores de DNA.

REFERENCES

Aggarwal RK, Udaykumar D, Handre PS, Sarkar A and Singh LI (2004) Isolation and characterization of six novel microsatellite markers for mulberry (*Morus indica*). **Molecular Ecology Notes 4**: 477-479.

Awasthi AK, Nagaraja GM, Naik GV, Sriramana K, Thangavelu K and Nagaraju J (2004) Genetic diversity and relationships in mulberry (genus Morus) as revealed by RAPD and ISSR marker assays. **BMC Genetics 5**: 1-18.

Bhat KV and Jarret RL (1995) Random amplified polymorphic DNA and genetic diversity in Indian *Musa* germplasm. **Genetic Resources and Crop Evolution 42**: 107-118.

Casas AM, Lgartua E, Balaguer G and Moreno MA (1999) Genetic diversity of *Prunus* root stocks analyzed by RAPD markers. **Euphytica 110**: 139-149.

Fotadar RK and Dandin SB (1998) Genetic divergence in the mulberry. **Canye Kexue 24**: 180-185.

Gomez PM, Sozzi GO, Perezi RS, Rubio M and Gradziel TM (2003) New approaches to *Prunus* tree crop breeding. **Food, Agriculture & Environment 1**: 52-63.

Krabel D, Vornam B and Herzog S (1998) PCR-based Random Amplification of genomic DNA from single pollen grains of tree. **Angew Botany 72**: 10-13.

Sharma A and Sharma MH (2000) Assessment of genetic diversity in a *Morus* germplasm collection using fluorescence based AFLP markers. **Theoretical and Applied Genetics 101**: 1049-1055.

Sneath PHA and Sokal RR (1973) **Numerical taxonomy**. San Francisco, Freeman, 573p.

Murray MG and Thompson WF (1980) Rapid isolation of high-molecular weight plant DNA. **Nucleic Acid Research 8**: 4321-4327.

Neale DB, Devey ME, Jermstad ISD, Ahuja MR, Alosi MC and Marshall KA (1992) Use of DNA markers in forest tree improvement research. **New Forests 6**: 391-407.

Rao MN, Jaya RS, Chunxian C, Huang S and Fred GG (2008) Characterization of zygotic and nucellar seedlings from sour orange-like citrus rootstock candidates using RAPD and EST-SSR markers. **Tree Genetics & Genomes 4**: 113-124.

Ravishankar KV, Anand L and Dinesh MR (2000) Assessment of genetic relatedness among mango cultivars of India using RAPD markers. **Journal of Horticulture Science and Biotechnology 75**: 198-201.

Treuren R and Hintum TJL (2003) Marker-assisted reduction of redundancy in germplasm collections: genetic and economic aspects. **Acta Horticulture 623**: 139-149.

Venkateswarlu M, Urs, SR Surendra BN, Shashidhar HE, Maheswaran M, Veeraiah TM and Sabitha MG (2006) A first genetic linkage map of mulberry (*Morus* spp.) using RAPD, ISSR, and SSR markers and pseudo testcross mapping strategy. **Tree Genetics & Genomes 3**: 15-24.

Vijayan K, Awasthi AK, Srivatsava PP and Saratchandra B (2004) Genetic analysis of Indian mulberry varieties through molecular markers. **Hereditas 141**: 8-14.

Vijayan K, Srivatsava PP, Nair CV, Awasthi AK, Tikader A, Sreenivasa B and Urs SR (2006) Molecular characterization and identification of markers associated with yield traits in mulberry using ISSR markers. **Plant Breeding 125**: 298-301.

Williams JKG, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acid Research 18**: 6531-6535.

Xu Y (2003) Developing marker-assisted selection strategies for breeding hybrid rice. **Plant Breeding Reviews 23**: 73-174.